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S/N: 10/033,129
8325-2001.30
Nucleic Acids Research, Vol. 19, No. 21 5901–5905

A thermodynamic study of unusually stable RNA and DNA hairpins

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Received August 12, 1991; Revised and Accepted October 11, 1991

ABSTRACT

About 70% of the RNA tetra-loop sequences identified in ribosomal RNAs from different organisms fall into either (UNCG) or (GNRA) families (where N = A, C, G, or U; and R = A or G). RNA hairpins with these loop sequences form unusually stable tetra-loop structures. We have studied the RNA hairpin GGAC(UUCG)GUCC and several sequence variants to determine the effect of changing the loop sequence and the loop-closing base pair on the thermodynamic stability of (UNCG) tetra-loops. The hairpin GGAG(CUUG)CUCC with the conserved loop G(CUUG)C was also unusually stable. W have determined melting temperatures (T_m), and obtained thermodynamic parameters for DNA hairpins with sequences analogous to stable RNA hairpins with (UNCG), C(GNRA)G, C(GAUA)G, and G(CUUG)C loops. DNA hairpins with (TTCG), (dUdUCG), and related sequences in the loop, unlike their RNA counterparts, did not form unusually stable hairpins. However, DNA hairpins with the consensus loop sequence C(GNRA)G were very stable compared to hairpins with C(TTTT)G or C(AAAA)G loops. The C(GATA)G and G(CTTG)C loops were also extra stable. The relative stabilities of the unusually stable DNA hairpins are similar to those observed for their RNA analogs.

INTRODUCTION

The primary structure of RNA molecules is unable to account for their numerous functions; RNA molecules usually fold into some specific three-dimensional structure. From x-ray crystallography studies of tRNA molecules it is evident that the majority of the interactions stabilizing the tertiary structure are also present in the secondary structure (1). Secondary structures determined by phylogenetic comparisons of 16S and 23S RNAs from various organisms show that hairpins are by far the most common structural motif (2). Despite the importance of hairpins in RNA secondary structure, until recently very little was known about the effect of the size and sequence of the loop on the stability of hairpins. From studies of a series of RNA hairpins containing only cytosines in the loop, it was concluded that the most stable loop contained six or seven nucleotides (3, 4). This seemed reasonable because the loops in tRNA were about this size. However, the predicted secondary structures of 16S and 23S RNAs from many organisms show that almost half of the phylogenetically proven hairpins have four nucleotides in the loop (5). To investigate this further, Groebe and Uhlenbeck (6) determined the thermal stabilities of fifteen RNA hairpins with the general sequence GGGAUAC(N_x)GUAUCCA (where N = A, C, or U and x = 3, 4, 5, 7, or 9). They concluded that loops of four or five nucleotides were the most stable and that the thermal stability of the hairpins did not depend significantly on the loop composition. Tuerk *et al.* (7) found that messenger RNAs with the sequence 5′...C(UUCG)G...3′ prevented reverse transcriptase from reading through, and that hairpin loops with this sequence were unusually stable. Two-dimensional NMR techniques were used to determine the structural basis for the unusual thermal stability of this hairpin (8–10). The first and last bases of the loop form a reverse wobble U·G base pair with G *syn*, and the amino group of the cytosine in the loop forms a hydrogen bond with a neighboring phosphate. These interactions within the four-base loop help explain the unusual stability of the hairpin.

Although the (UUCG) loop is a commonly-occurring loop sequence in 16S and 23S RNAs, there are other loop sequences such as (GAAA), (GCAA), (GAGA), (GUGA) and (GGAA) which occur even more frequently in the secondary structures (5). Haney and Uhlenbeck (personal communication) measured the thermal stabilities of RNA hairpins having loops with the consensus sequence (GNRA) and compared them with RNA hairpins having (UUUU) and (AAAA) loops. They found that the hairpins with (GNRA) loop sequences were also unusually stable.

It is important to learn if DNA, like RNA, can form unusually stable hairpins, and if so, whether the same sequences are extra-stable. Although the most common secondary structure of DNA is the double helix, there are several stages during the cell cycle, for example, replication, transcription, etc. when segments of the DNA may be single-stranded and could adopt other secondary

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res such as hairpins. Palindromic sequences that can form ns are often found at origins of replication and in regions ed for the control of gene expression. If hairpins are formed transiently in such regions, they could act as recognition and/or binding sites for proteins involved in the regulation of replication and gene expression. Increased supercoiling was shown to result in the formation of hairpins in covalently-closed DNA molecules (11, 12). Structural motifs such as hairpins might also play an important role in the structure and function of single-stranded DNA viral genomes.

Information about the dependence of the stability of DNA hairpins on the size and sequence of their loops has been as meager as that for RNA hairpins. Blommers *et al.* (13) showed that DNA hairpins with the sequence ATCCTA(T_n)TAGGAT (where n = 3, 4, 5, 6, or 7) showed maximum stability when the loop consisted of four or five nucleotides. Senior *et al.* (14) determined the relative stabilities of four DNA hairpins with the sequence CGAACG(X_4)CGTTCG, where x = A, C, G, or T. The order of stability for these four DNA hairpins was $T_{loop} > C_{loop} > G_{loop} > A_{loop}$.

We have focused on a comparison of thermodynamic parameters for extra-stable RNA hairpins [(UNCG), C(GNRA)G, C(GAUA)G, and G(CUUG)C] with the corresponding DNA hairpins. We find that extra-stable RNA hairpins do not necessarily translate into extra-stable DNA hairpins.

MATERIALS AND METHODS

IA template molecules for synthesis of the RNA hairpins, ai.. ne DNA hairpin molecules were synthesized by the phosphoramidite method on an automated DNA synthesizer (Applied Biosystems, Inc.). After deprotection, the DNA oligomers were purified by polyacrylamide gel electrophoresis. The RNA hairpins were synthesized using T7 RNA polymerase (15) and purified by polyacrylamide gel electrophoresis. The sequences of the RNA molecules were determined enzymatically.

To determine if hairpins and single strands were the only species present in our studies, the absorbance melting profile for each molecule was measured over at least a hundred-fold range in nucleic acid concentration (1 mM to 10 μM strands). The similarity of these melting profiles for most of the molecules indicated that the species involved were unimolecular. For some of the molecules measured at the highest ionic strength (1 M NaCl, 0.01 M Na^+(phosphate) and 0.1 mM EDTA), the melting profiles at ten-fold and one hundred-fold the usual nucleic acid concentration indicated the presence of a small amount of a second species (most likely the dimer duplex). At the nucleic acid concentrations used to determine the thermodynamic parameters there was no indication of the presence of any species other than the hairpin and single strand.

For the melting profiles, the DNA and RNA stock solutions were extensively dialyzed, first against 0.01 M EDTA, 0.5 M NaCl, and 0.01 M Na^+(phosphate), second against 0.5 M NaCl, and 0.01 M Na^+(phosphate), third against 0.01 M N.. osphate), all at pH 7, and finally against double-distilled w.. or each melting profile, a small volume of the oligomer s.. vas dried in a speed-vac and the sample was then dissolved in buffer. The buffers were at pH 7 ± 0.1 and contained either 1 M NaCl, 0.01 M Na^+(phosphate) and 0.1 mM EDTA, or 0.01M Na^+(phosphate) and 0.1 mM EDTA. UV absorbance melting profiles at 260 nm were obtained using a Gilford 250 Spectrophotometer and 1 cm pathlength cuvettes. The samples were rapidly heated to above 90°C for a few minutes and then cooled to 1°C to begin the experiment. During the melting experiment the heating rate was 0.5°C/min and was controlled by a Gilford 2527 Thermo-programmer. The data shown represent the average of at least nine independent melting profiles for each hairpin. Thermodynamic parameters were determined from plots of fraction single strand vs. temperature by standard methods (16). The variation in the parameters are within ± 1°C for the T_m, ± 0.2 kcal/mol for $\Delta G°_{37}$, and ± 5% for $\Delta H°$ and $\Delta S°$. The variation in the values shown for $\Delta H°$ and $\Delta S°$ for four of the DNA hairpins {GGAG(TTTG)CTCC, GGAG(TTCG)CTCC, GATC(AAAA)GATC, and GATC(GCAA)GATC} are slightly greater, being within ± 7.5% instead of within ± 5%. Additional significant figures are given for $\Delta H°$ and $\Delta S°$ to allow more accurate calculation of $\Delta G°$ at various temperatures.

RESULTS AND DISCUSSION

RNA Hairpins: the (UNCG) family

Table 1 presents thermodynamic data for (UNCG) RNA hairpins and the corresponding (TTCG) DNA hairpins in 0.01 M Na^+(phosphate), pH 7 buffer. The RNA hairpins with (UUCG) loops (whether the loop-closing base pair was C·G or G·C) were considerably more stable than the corresponding hairpins with (UUUG). This is in agreement with the work of Tuerk *et al.* (7) on the hairpin GGGC(UUCG)GCCUUAU. In addition, we obtained thermodynamic parameters for hairpins with C(UUUU)G and G(UUUU)C loops. The C(UUUU)G loop was the most stable among RNA C(AAAA)G, C(CCCC)G, or C(UUUU)G loops (6); we therefore consider hairpins that are significantly more stable than the corresponding (UUUU) hairpin as being unusually stable. The hairpin with the C(UUCG)G loop (T_m = 71.7°C) was considerably more stable thermally than the hairpin with the C(UUUG)G loop (T_m = 64.0°C), which in turn was significantly more stable than the hairpin with the C(UUUU)G loop (T_m = 60.4°C).

Changing the loop-closing base pair from C·G to G·C resulted in significant decreases in the T_m (Table 1). However, the hairpin with the loop G(UUCG)C (T_m = 60.1°C) was still considerably more stable than those with the loop G(UUUG)C (T_m = 51.1°C) or the loop G(UUUU)C (T_m = 51.5°C). Thus (UUCG) stabilizes a hairpin with either C·G or G·C loop-closing base pairs, although C·G loop-closing base pairs are preferred in nature as shown by the frequency of their occurrence in secondary structures predicted for ribosomal RNAs (5). The factors that contribute to the difference in stability caused by changing the loop-closing base pair include: differences in the stem nearest-neighbors, and thus their contributions to the stability of the stem, differences in stacking of loop nucleotides on the closing base pair, and changes in structure of the loop. The effect of stem stability and of stacking of the end bases of the loop on the decrease in stability can be seen by comparing the thermodynamic parameters of the ...C(UUUU)G... and ...G(UUUU)C... RNA hairpins. We note a 9°C drop in T_m and a 1.3 kcal/mol increase in free energy at 37°C for the G·C closing base pair vs. the C·G pair. Since (UUUU) is a symmetrical loop sequence, these differences between the two hairpins will largely be due to the direct effect of the loop-closing base pair on the stem and on the end bases of the loop. The

somewhat similar decrease in T_m on changing the loop-closing base pair from C·G to G·C for (UUUU) and (UUCG) hairpins indicates only a minor disturbance of the loop structure.

The hairpin with the C(GCUU)G loop was not significantly more stable than the hairpin with the C(UUUU)G loop. This indicates that the 5′ to 3′ directionality of the bases in the loop is critical for the formation of the unusually stable loop structure. The hairpin with the loop G(GCUU)C, where the same bases are stacked on each other as in the C(UUCG)G loop (but in different 5′ to 3′ orientations), was also not unusually stable.

The sequence UACG occurs as a four-base loop in secondary structures of ribosomal RNAs with about the same frequency as does UUCG (5); Table 1 shows that it was also extra stable. This is consistent with the structure of the GGAC(UUCG)GUCC hairpin (8, 10). The second U in the C(UUCG)G loop is not involved in any base-base interactions (unlike the first U and the G), or base-backbone interactions (unlike the C of the loop), and would therefore be a likely candidate for sequence variability while still maintaining the overall stable loop conformation.

The sequence GAAA belongs to the most common tetra-loop family in ribosomal RNAs; it is also extra stable. Table 1 shows that although the loop C(GAAA)G is not as stable as UUCG or UACG, it is significantly more stable than UUUU. This is in agreement with the data of Haney and Uhlenbeck (personal communication).

DNA Hairpins: the (TNCG) family

The right half of Table 1 shows the T_m and thermodynamic parameters of DNA hairpins, many of which have sequences analogous to the RNA hairpins studied. The melting temperatures of all the DNA hairpins (with the exception of the ...G(C-TTG)C... hairpin), were much lower than the corresponding RNA hairpins. This might be expected since a comparison of the free energies for formation of DNA nearest-neighbor base pairs (17) and RNA nearest-neighbor base pairs (18) indicates that DNA stems will be less stable than RNA stems of the same sequence. It is clear from Table 1 that DNA hairpins with loop sequences analogous to the RNA (UUCG) or related loop sequences did not form unusually stable hairpins. In fact, all the DNA hairpins having the same stem sequence (excluding ...G(C-TTG)C...) had similar melting temperatures and thermodynamic parameters (Table 1). To determine if the methyl group on the thymines in the loop prevented the stable tetra-loop structure from forming, we synthesized hairpins with either one or both loop thymidine residues replaced by deoxyuridine (dU). None of these hairpins showed an increased stability over that of the (TTCG) hairpin (see also Table 2). Thus the (TTCG) DNA hairpin is not extra-stable, and it is not the thymine methyls which prevent the stability.

Sakata *et al.* (9) showed that the hairpin r{UG-AGC}d(UUCG)r{GCUC} was not unusually stable. The stem is A-form with C3′-endo ribonucleotides, therefore it must be the deoxyribose sugars in the loop that prevent the formation of the unusually stable hairpin. The sugars of the central two nucleotides of the (UUCG) loop are in the C2′-endo conformation, but all the other nucleotides are C3′-endo (8, 10). Deoxyribose sugars can easily form either of these conformations; thus the 2′-hydroxyl groups themselves must have a role in the stabilization of this unusually stable loop structure. Specific tertiary interactions involving 2′-hydroxyl groups have been shown to be very important in the binding of a ribozyme to its substrate (19).

Table 1. RNA and DNA Hairpins in 0.01 M sodium phosphate, 0.1 mM EDTA, at pH 7.

RNA GGAX(NNNN)X'UCC Hairpin Parameters					DNA GGAX(NNNN)X'TCC Hairpin Parameters				
RNA Loop Sequence	Tm (°C)	ΔH° (kcal/mol)	ΔS° (e.u.)	ΔG°(37) (kcal/mol)	DNA Loop Sequence	Tm (°C)	ΔH° (kcal/mol)	ΔS° (e.u.)	ΔG°(37) (kcal/mol)
C(UUCG)G	71.7	-56.5	-163.9	-5.7	C(TTCG)G	53.1	-32.7	-100.1	-1.6
C(UUUG)G	64.0	-47.4	-140.6	-3.8	C(TTTG)G	52.4	-32.9	-101.0	-1.6
C(UUUU)G	60.4	-42.7	-127.9	-3.0	C(TTTT)G	53.0	-33.4	-102.5	-1.6
C(UACG)G	69.3	-54.9	-160.4	-5.2	C(dUdUCG)G	52.3	-33.5	-103.0	-1.6
C(GCUU)G	62.2	-42.7	-127.3	-3.2	C(GCTT)G	51.4	-30.8	-94.9	-1.4
C(GAAA)G	65.9	-49.1	-145.0	-4.2					
G(UUCG)C	60.1	-48.6	-145.7	-3.4	G(TTCG)C	44.7	-31.2	-98.0	-0.8
G(UUUG)C	51.1	-39.2	-121.0	-1.7	G(TTTG)C	44.2	-31.3	-98.6	-0.7
G(UUUU)C	51.5	-38.2	-117.8	-1.7					
G(CUUG)C	62.4	-47.5	-141.6	-3.6	G(CTTG)C	64.2	-41.1	-121.8	-3.3
G(GCUU)C*	52.3	-	-	-1.7					

* This hairpin differs from the rest in that it has an additional (unpaired) G at the 5′ end.

Table 2. DNA GATC(NNNN)GATC hairpins in 1.0 M sodium chloride, 0.01 M sodium phosphate, 0.1 mM EDTA, at pH 7.

DNA Loop Sequence	Tm (°C)	ΔH° (kcal/mol)	ΔS° (e.u.)	ΔG°(37) (kcal/mol)
C(TTTT)G	51.8	-30.7	-94.4	-1.4
C(AAAA)G	45.2	-23.1	-72.5	-0.6
C(TTCG)G	50.2	-29.6	-91.7	-1.2
C(dUdUCG)G	51.4	-30.2	-93.0	-1.3
C(TdUCG)G	50.9	-28.4	-87.6	-1.2
C(dUTCG)G	50.9	-29.6	-91.5	-1.3
C(GAAA)G	60.5	-30.9	-92.8	-2.2
C(GCAA)G	63.5	-33.4	-99.2	-2.6
C(GTAA)G	64.6	-33.7	-99.9	-2.7
C(GATA)G	62.9	-34.1	-101.5	-2.6

RNA Hairpin: the G(CUUG)C loop

The G(CUUG)C loop is not a common tetra-loop sequence. However, phylogenetic comparisons of many 16S ribosomal RNAs showed that hairpins with this loop sequence are highly conserved at position 83 (5). The (CUUG) loop is almost always closed by G·C, unlike the (GNRA) and (UNCG) loops which are usually closed by a C·G base pair. The RNA ...G(CUUG)C... hairpin was considerably more stable than the ...G(UUUU)C... hairpin. The unusual stability of the G(CUUG)C loop is yet another example of a phylogenetically highly conserved RNA hairpin loop also being thermodynamically very stable. The DNA with the analogous loop sequence G(CTTG)C was very stable compared with any of the DNA hairpins listed in Table 1. The effect on the stability of either the RNA or the DNA hairpin upon changing the loop-closing base pair from G·C to C·G has yet to be determined.

DNA Hairpins: the (GNRA) family

Uhlenbeck and Haney (personal communication) have found that RNA hairpins with C(UUCG)G and C(GNRA)G loops were significantly more stable than similar hairpins with C(UUUU)G or C(AAAA)G loops. Our own studies (unpublished) showed that hairpins with A(GAAA)U loops had higher melting temperatures than hairpins with identical stem sequences, but with A(UUUU)U loops. We have obtained thermodynamic parameters for DNA hairpins with loop sequences identical to the naturally-occurring RNA (GNRA) stable tetra-loop sequences. Table 2 shows that the DNA hairpins with the consensus loop sequence C(GNRA)G were considerably more stable than hairpins with the same stem but with C(TTTT)G or C(AAAA)G loops. The unusual stability of these loops is not simply because C(GNRA)G loops are purine-rich since the all-purine C(AAAA)G loop is in fact less stabilizing than the C(TTTT)G loop, and considerably less than the C(GNRA)G loops.

The DNA hairpin with the C(GAGA)G loop also had a T_m of about 60°C (data not shown). However, the melting profile indicated the presence of a small amount of the dimer duplex even at the nucleic acid concentrations used for the rest of the molecules. This suggests that the GAGA/GAGA internal loop consisting of all G3A mismatches was significantly more stable than the internal loops that could form for the other molecules studied here. This is consistent with the findings of SantaLucia et al., (20) that neighboring G3A mismatches are unususally stable.

The DNA hairpins showed a pattern of relative stability similar to that observed for the RNA hairpins with identical loop sequences; thereby suggesting that the conformations of these C(GNRA)G loops are similar in both RNA and DNA. The order of stability of RNA loop sequences did not necessarily correspond with the frequency with which they occur in ribosomal RNAs (Haney and Uhlenbeck; personal communication). For example, an RNA hairpin with the most frequently-occurring loop sequence C(GAAA)G had a lower melting temperature than RNA hairpins with less frequently-occurring loops such as C(GCAA)G, C(GGAA)G and C(GUAA)G.

The consensus sequence (GNRA) has a purine as the third nucleotide in the loop, and in ribosomal RNAs, (GAUA) is not a common loop sequence (5). However, both the RNA hairpin with the C(GAUA)G loop (Haney and Uhlenbeck, personal communication), and the DNA hairpin with the same loop (Table 2), had melting temperatures higher than the corresponding hairpins with the C(GAAA)G loop. Thus, secondary structure prediction programs which assign favorable free energy bonuses to loops that fall into the (UNCG) and (GNRA) families (21) may need to add other tetra-loops such as C(GAUA)G and G(CUUG)C to their bonus list.

NMR studies of C(GNRA)G RNA hairpins (22, 23) provide some explanation of their extra stability. The hairpin is closed by an unusual G·A base pair; the N7 of the third base (which must be a purine) is involved in a hydrogen bond; and there is a G amino−phosphate hydrogen bond (23). The second base (which can be any base) is not involved in any specific contacts with other bases of the loop. The overall folding of the loop is very similar to that found in C(UNCG)G loops.

SUMMARY AND CONCLUSION

RNA hairpins with (UUCG) loops are considerably more stable than the corresponding hairpins with (UUUG) or (UUUU) loops, whether the loop-closing base pair is C·G or G·C. Some, though certainly not all, of the difference in stability between the hairpin with the C(UUCG)G and that with the G(UUCG)C loop can be accounted for by the differences in their nearest-neighbor interactions in the stem. Whether the conformation of the loop is significantly different in these two loops remains to be determined.

The 5′ to 3′ directionality of the loop nucleotides is important for the unusually stable (UNCG) loop structure since neither the C(GCUU)G nor the G(GCUU)C loop resulted in an unusually stable hairpin. As one might predict from phylogenetic comparisons of ribosomal RNAs, as well as from the solution structure determined by 2D NMR techniques, the ...C(UACG)G... hairpin was about as stable as the ...C(UUCG)G... hairpin; this indicates that the unusually stable (UUCG) loop conformation permits variation of the base in the second position of the loop.

The RNA hairpin with the G(CUUG)C loop, a highly conserved loop at position 83 of 16S ribosomal RNA, was also unusually stable, thus providing another example of a possible correlation between phylogenetic conservation and thermodynamic stability.

DNA hairpins with a C(TTCG)G loop and variants of this loop sequence did not form extra-stable tetra-loop structures. This is not because of the substitution of thymine for uracil in the DNA loop, but is caused by the presence of deoxyribose sugars in the loop. However, DNA hairpins with a G(CTTG)C loop, a C(G-ATA)G loop, and the C(GNRA)G family of loops were extra stable. We do not know the structural explanation for why these DNA hairpins, but not the DNA analogs of the (UNCG) family, form unusually stable hairpins.

ACKNOWLEDGEMENTS

We thank Mr. David Koh for synthesizing the DNA molecules used in this study, and Ms. Barbara Dengler for managing the laboratory. We are grateful to Ms. Kristina Downing and Ms. Arlene Lee, both undergraduate students in the laboratory, for their help with some of the preliminary experiments, and to Dr. John Jaeger and Dr. Gabriele Varani for their helpful suggestions. S.Y.L. was an undergraduate student in the laboratory. This research was supported in part by the National Institutes of Health grant GM 10840 and the Department of Energy, Office of Energy Research, Office of Health and Environmental Research grant DE-FG03-86ER60406.

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